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TITLE OF INVENTION

USE OF SPECIFIC HYBRID PROMOTERS FOR CONTROLLING TISSUE EXPRESSION

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

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17. <input checked="" type="checkbox"/> The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445 (a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... \$1000.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... \$860.00 International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... \$710.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... \$690.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... \$100.00 <b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		CALCULATIONS PTO use only			
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USE OF SPECIFIC HYBRID PROMOTERS  
FOR CONTROLLING TISSUE EXPRESSION

5           The present invention relates to the field of biology, and in particular the  
field of the regulation of the expression of genes. It describes in particular new constructs  
and new vectors allowing a targeted and high expression of genes. The present invention  
can be used in many fields, and in particular for the production of recombinant proteins, for  
the creation of transgenic animal models, for the creation of cell lines, for the development  
10 of screening tests, or in gene and cell therapy.

          The possibility of controlling and directing the expression of genes  
constitutes a very important stake in the development of biotechnologies. In vitro, it can  
make it possible to improve the conditions for producing recombinant proteins using  
particular populations of cells. Still in vitro, it can allow the detection or the revealing of the  
15 presence of specific populations of cells in a sample, and also the testing of the properties  
of a product or the regulation of a gene in a specific population of cells. The control of the  
expression of genes is also very important for therapeutic approaches ex vivo or in vivo, in  
which the possibility of selectively controlling the production of a therapeutic molecule is  
essential. Indeed, depending on the applications, depending on the gene to be transferred,  
20 it is important to be able to target only certain tissues or certain parts of an organism in  
order to concentrate the therapeutic effect and to limit dissemination and side effects.

          The targeting of the expression of a given nucleic acid may be achieved  
according to various approaches. A first approach consists, for example, in using transfer  
agents or vectors exhibiting a given cell specificity. However, the specificity conferred by  
25 this type of vectors is generally fairly rough and does not allow targeting of precise  
populations of cells. Another approach consists in using expression signals specific for  
certain cell types. In this regard, so-called "specific" promoters have been described in the  
literature, such as the promoter of the genes encoding pyruvate kinase, villin, GFAP, the  
promoter of the fatty acid-binding intestinal protein, the  $\alpha$ -actin promoter of the smooth  
30 muscle cells, the SM22 promoter or alternatively the promoter of the human albumin gene  
for example. However, while these promoters exhibit tissue specificity, they also exhibit, in  
return, a relatively low strength. Thus, the great majority of these promoters possess levels  
of activity which are well below those of so-called "strong" promoters, generally by a factor  
of between at least 10 and 100. In addition, it is generally considered that the specificity of  
35 a promoter is inversely proportional to its strength and that the higher the strength the  
higher the level of nonspecific activity.

          It would therefore be particularly advantageous to be able to have  
promoters which are both specific to certain tissues and strong. The subject of the present

invention is precisely to provide new constructs allowing the high and targeted expression of genes.

The invention describes in particular new chimeric promoters allowing a high and specific expression of the smooth muscle cells. The invention also describes  
5 vectors containing such promoters and their use for the transfer of genes into cells in vitro, ex vivo and in vivo. The constructs of the invention make it possible, for the first time, to combine opposing properties, namely a high selectivity and a high transcriptional activity. The invention thus offers a particularly efficient means for the targeting of the expression of genes in smooth muscle cells, in vivo or in vitro, and for the regulation of this  
10 expression.

The invention is based more particularly on the construction of chimeric (or hybrid) promoters comprising regions of different origin and function. More particularly, a first subject of the invention consists in a hybrid promoter comprising:

- all or part of the enhancer region of a strong and ubiquitous  
15 promoter/enhancer, and
- a promoter region allowing specific expression in the smooth muscle cells.

The combination of enhancer regions and of promoters has already been described in the prior art. Thus, it is known, for example, to couple the CMV enhancer  
20 region with nonspecific promoters such as the promoter of the chicken  $\beta$ -actin gene (WO96/13597) in order to increase their strength. However, such constructs have not been described or suggested with the aim of attempting to obtain a high expression specific to the smooth muscle cells. The invention is partly based on the selection and the combination of particular "enhancer" elements and of particular "promoter" elements. The  
25 invention is also based on the demonstration that this combination of elements makes it possible to obtain an expression at high levels, without affecting the selectivity of the promoter for the target cells of the smooth muscle. The invention therefore provides particularly advantageous constructs since they allow targeted production with high levels of molecules in the smooth muscle cells. In addition, the present application also shows  
30 that these constructs may be used both in vitro and in vivo.

In the hybrid promoters according to the invention, the enhancer region and the promoter region are functionally combined, that is to say such that the enhancer region exerts a stimulating activity on the activity of the promoter region. Generally, these two regions are therefore genetically linked and are sufficiently close to each other to allow  
35 the enhancer region to activate the promoter region. Preferably, the distance separating the enhancer region and the promoter region is less than 1 kb, more preferably less than 500 bp. In the particularly preferred constructs according to the invention, these two regions are less than 400 bp apart, more preferably less than 200 bp. In addition, as shown in the examples, the respective orientation of the two regions has no significant

influence on the activity of the hybrid promoters of the invention. As a result, the enhancer region may be positioned in the same orientation or in the opposite orientation relative to the direction of the transcription of the promoter region.

In a preferred embodiment, the enhancer region is chosen from the  
 5 enhancer region of the cytomegalovirus immediate-early (CMV-IE) gene, the enhancer region of the rous sarcoma virus LTR (RSV-LTR), the enhancer region of the SV40 virus, and the enhancer region of the EF1 $\alpha$  gene. More preferably, in the hybrid promoters of the invention, the enhancer region is the enhancer region of the immediate-early gene of the cytomegalovirus (CMV-IE), preferably of the human cytomegalovirus (hCMV-IE). A  
 10 particular enhancer region consists, for example, of fragment -522 to -63 of the hCMV-IE gene, or of any fragment comprising at least part of it and exhibiting an enhancer activity.

As regards the promoter region, there is advantageously used for carrying out the invention a region comprising all or part of the promoter of the gene encoding the  $\alpha$ -actin of smooth muscle cells (SMact) or of the SM22 gene. The promoter of these genes  
 15 has been described for its smooth muscle cell-specific character (see in particular Ueyama H. et al., *Mol. Cell. Biol.*, **4** (1984) 1073-1078; Solway J. et al., *J. Biol. Chem.*, **270** (1995) 13460-13469).

A first particularly advantageous variant of the present invention consists of a hybrid promoter comprising:

- 20 - all or part of the enhancer region of the human cytomegalovirus immediate-early (hCMV-IE) gene, and
- all or part of the promoter of the gene encoding the  $\alpha$ -actin of smooth muscle cells (SMact), preferably of the human SMact gene.

A second particularly advantageous variant of the present invention  
 25 consists of a hybrid promoter comprising:

- all or part of the enhancer region of the human cytomegalovirus immediate-early (hCMV-IE) gene, and
- all or part of the promoter of the SM22 gene, preferably of the mouse SM22 alpha gene.

30 Moreover, in a specific embodiment of the invention, the promoter region used is a chimeric region comprising a basal promoter and a sequence conferring tissue specificity, said sequence being derived from the SMact promoter or from the SM22 promoter, or from a combination of both. In this embodiment, the basal promoter may be a "minimal" promoter, that is to say comprising only the sequences essential for the  
 35 transcriptional promoter activity (for example a TATA box). This basal promoter may be the actual basal promoter of the SMact or SM22 gene, or of a heterologous origin ( $\beta$ -globin, HSV-TK, SV40 or EF1- $\alpha$  for example). The sequence conferring tissue specificity advantageously comprises part of the sequence of the SMact promoter (R.T. Shimizu et al. *J. Biol. Chem.* **270** (1995) 7634-7643) and/or of the SM22 promoter

(L. Li et al. *J. Cell. Biol.* **132** (1996) 849-859; S. Kim et al. *J. Clin. Invest.* **100** (1997) 1006-1014; Kemp et al., *Biochem. J.* **310** (1995) 1037-1043).

A particular type of hybrid promoter according to the invention therefore comprises:

- 5                               - all or part of the enhancer region of a strong and ubiquitous promoter/enhancer,
- a basal promoter, and
- a sequence conferring tissue specificity comprising all or part of the SMact promoter and/or of the SM22 promoter.

10                           For the construction of the hybrid promoters of the invention, molecular biology techniques known to persons skilled in the art may be used. Thus, the enhancer region and the promoter region (including the basal promoter and the sequence conferring tissue specificity) may be isolated by conventional techniques from nucleic acid libraries or from the total cellular DNA, for example by amplification by means of specific probes.

15   These fragments may also be synthesized artificially using the sequence information available in the prior art. When these fragments are obtained, they can be easily combined with one another by means of ligases and other restriction enzymes, in order to generate hybrid promoters of the invention. In addition, these fragments may be modified by digestion, mutation, insertion or addition of base pairs, either with the aim of facilitating

20   their cloning, or with the aim of modifying their functional properties. Moreover, as indicated above, the fragments may be combined directly with one another or, on the contrary, separated by base pairs having no significant influence on the activity of the hybrid promoter.

                              The hybrid promoters of the invention thus possess the capacity to

25   express specifically a nucleic acid of interest in the smooth muscle cells. The "specific" character of the expression means that the activity of the promoter is significantly much higher in the smooth muscle cells. Although a nonspecific expression may exist in other cells, the corresponding level of activity remains generally very low (negligible) compared with that observed in the smooth muscle cells, generally lower by a factor of at least 10.

30   The results presented in the examples show, in this regard, a difference in expression which may be up to a factor of 140, which reflects the high selectivity of the promoters of the invention. In this regard, the results presented also show a high specificity towards the smooth muscle cells since no expression was detected in the endothelial cells which exist in the vicinity, in the blood vessel, in particular the artery. The results presented in the

35   examples also show that the strength of the promoters of the invention is much higher than that of the nonhybrid specific promoters, it being possible for the difference to exceed a factor of 100. These features therefore illustrate the advantageous and unexpected properties of the hybrid promoters of the invention, in terms of strength and specificity, for the expression of nucleic acids of interest in the smooth muscle cells.

- the transcription factors, such as for example natural or chimeric receptors, comprising a DNA-binding domain, a ligand-binding domain, a transcription activating or inhibiting domain, such as for example the tetR-NLS-VP16 fusion proteins, the fusion proteins derived from the estrogen receptors,

Adenoviruses are linear double-stranded DNA viruses of about 36 (kilobases) kb in size. Various serotypes exist whose structure and properties vary somewhat but which exhibit a comparable genetic organization. More particularly, the recombinant adenoviruses may be of human or animal origin. As regards the adenoviruses of human origin, there may be preferably mentioned those classified in group C, in particular the adenoviruses of type 2 (Ad2), 5 (Ad5), 7 (Ad7) or 12 (Ad12).



Among the various adenoviruses of animal origin, there may be preferably mentioned the adenoviruses of canine origin, and in particular all the strains of the CAV2 adenoviruses [manhattan or A26/61 strain (ATCC VR-800) for example]. Other adenoviruses of animal origin are cited in particular in Application WO94/26914 incorporated into the present application by reference.

The genome of the adenoviruses comprises in particular an inverted repeat sequence (ITR) at each end, an encapsidation sequence (Psi), early genes and late genes. The principal early genes are contained in the E1, E2, E3 and E4 regions. Among these, the genes contained in the E1 region in particular are necessary for viral propagation. The principal late genes are contained in the L1 to L5 regions. The genome of the adenovirus Ad5 has been completely sequenced and is accessible on database (see in particular Genebank M73260). Likewise, portions or even the whole of other adenoviral genomes (Ad2, Ad7, Ad12 and the like) have also been sequenced.

For their use as recombinant vectors, various constructs derived from adenoviruses have been prepared, incorporating various therapeutic genes. In each of these constructs, the adenovirus was modified so as to make it incapable of replicating in the cell infected. Thus, the constructs described in the prior art are adenoviruses deleted for the E1 region, which is essential for viral replication, at the level of which the heterologous DNA sequences are inserted (Levrero et al., Gene 101 (1991) 195; Gosh-Choudhury et al., Gene 50 (1986) 161). Moreover, in order to improve the properties of the vector, it has been proposed to create other deletions or modifications in the genome of the adenovirus. Thus, a heat-sensitive point mutation was introduced into the ts125 mutant, making it possible to inactivate the 72 kDa DNA-binding protein (DBP) (Van der Vliet et al., 1975). Other vectors comprise a deletion of another region essential for replication and/or for viral propagation, the E4 region. The E4 region is indeed involved in the regulation of the expression of the late genes, in the stability of the late nuclear RNAs, in the extinction of the expression of the host cell proteins and in the efficiency of the replication of the viral DNA. Adenoviral vectors in which the E1 and E4 regions are deleted therefore possess a transcription background noise and a highly reduced expression of viral genes. Such vectors have been described not example in applications WO94/28152, WO95/02697, WO96/22378). In addition, vectors carrying a modification at the level of the IVa2 gene have also been described (WO96/10088).

In a preferred embodiment of the invention, the recombinant adenovirus is a group C human adenovirus. More preferably, it is an adenovirus Ad2 or Ad5.

Advantageously, the recombinant adenovirus used within the framework of the invention comprises a deletion in the E1 region of its genome. Still more particularly, it comprises a deletion for the E1a and E1b regions. By way of precise example, there may be mentioned deletions affecting nucleotides 454-3328; 382-3446 or 357-4020 (with reference to the genome of Ad5).

According to a preferred variant, the recombinant adenovirus used within the framework of the invention comprises, in addition, a deletion in the E4 region of its genome. More particularly, the deletion in the E4 region affects all the open reading frames. There may also be mentioned, by way of precise example, the deletions  
5 33466-35535 or 33093-35535. Other types of deletions in the E4 region are described in Applications WO95/02697 and WO96/22378, incorporated into the present application by reference.

The expression cassette may be inserted at various sites of the recombinant genome. It may be inserted at the level of the E1, E3 or E4 region, as a  
10 replacement for the deleted sequences or as a surplus. It may also be inserted at any other site, outside the sequences which are necessary in cis for the production of viruses (ITR sequences and encapsidation sequence).

The recombinant adenoviruses are produced in an encapsidation line, that is to say a line of cells capable of trans-complementing one or more of the functions  
15 deficient in the recombinant adenoviral genome. Among the encapsidation lines known to persons skilled in the art, there may be mentioned for example the 293 line into which part of the adenovirus genome has been integrated. More precisely, the 293 line is a human embryonic kidney cell line containing the left end (about 11-12%) of the adenovirus genome serotype 5 (Ad5), comprising the left ITR, the encapsidation region, the E1 region,  
20 including E1a and E1b, the region encoding the protein pIX and part of the region encoding the protein pIVa2. This line is capable of trans-complementing recombinant adenoviruses which are defective for the E1 region, that is to say lacking all or part of the E1 region, and of producing viral stocks having high titers. This line is also capable of producing, at permissive temperature (32°C), virus stocks comprising, in addition, the  
25 heat-sensitive E2 mutation. Other cell lines capable of complementing the E1 region have been described, based in particular on human lung carcinoma cells A549 (WO94/28152) or on human retinoblasts (Hum. Gen. Ther. (1996) 215). Moreover, lines capable of trans-complementing several functions of the adenovirus have also been described. In particular, there may be mentioned lines complementing the E1 and E4 regions (Yeh et  
30 al., J. Virol. Vol. 70 (1996) pp 559-565; Cancer Gen. Ther. 2 (1995) 322; Krougliak et al., Hum. Gen. Ther. 6 (1995) 1575) and lines complementing the E1 and E2 regions (WO94/28152, WO95/02697, WO95/27071).

Recombinant adenoviruses are usually produced by introducing viral DNA into the encapsidation line, followed by lysis of the cells after about 2 or 3 days (the  
35 kinetics of the adenoviral cycle being 24 to 36 hours). For carrying out the method, the viral DNA introduced may be the complete recombinant viral genome, optionally constructed in a bacterium (WO96/25506) or in a yeast (WO95/03400), transfected into the cells. It may also be a recombinant virus used to infect the encapsidation line. The viral DNA may also be introduced in the form of fragments each containing part of the

recombinant viral genome and a region of homology which makes it possible, after introduction into the encapsidation cell, to reconstitute the recombinant viral genome by homologous recombination between the various fragments.

After lysis of the cells, the recombinant viral particles are isolated by cesium chloride gradient centrifugation. An alternative method has been described in Patent FR96:08164 incorporated into the present application by reference.

The invention also relates to a composition comprising a vector as defined above and a chemical or biochemical transfer agent. The term "chemical or biochemical transfer agent" is understood to mean any compound (i.e. other than a recombinant virus) which facilitates the penetration of a nucleic acid into a cell. It may represent cationic nonviral agents such as cationic lipids, peptides, polymers (Polyethylene Imine, Polylysine), nanoparticles; or noncationic nonviral agents such as noncationic liposomes, noncationic nanoparticles or polymers. Such agents are well known to persons skilled in the art.

The invention also relates to a composition comprising a recombinant virus as defined above and a physiologically acceptable vehicle.

The invention also relates to a pharmaceutical composition comprising a vector as described above. The pharmaceutical compositions of the invention may be formulated for administration by the topical, oral, parenteral, intranasal, intravenous, intramuscular, subcutaneous, intraocular or transdermal route and the like.

Preferably, the pharmaceutical composition contains pharmaceutically acceptable vehicles for an injectable formulation, in particular for an intravascular injection or an injection into the smooth muscle tissues. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like, or mixtures of such salts), or dry, in particular freeze-dried, compositions which, upon addition, depending on the case, of sterilized water or of physiological saline, allow the constitution of injectable solutions. Other excipients may be used, such as for example a hydrogel. This hydrogel may be prepared from any biocompatible and noncytotoxic (homo- or hetero-) polymer. Such polymers have for example been described in Application WO93/08845. Some of them, such as in particular those obtained from ethylene and/or propylene oxide are commercially available. The use of a hydrogel is particularly advantageous for transferring nucleic acids into the vascular walls, and in particular into the smooth muscle cells of the vascular walls. The doses used for the injection may be adjusted as a function of various parameters, and in particular as a function of the mode of administration used, the aim pursued (labeling, pathology, screening and the like), the gene to be expressed, or alternatively the desired duration of expression. In general, the recombinant viruses according to the invention are formulated and administered in the form of doses of between  $10^4$  and  $10^{14}$  pfu, and preferably  $10^6$  to  $10^{10}$  pfu. The term pfu ("plaque forming unit") corresponds to the infectivity of a viral

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### LEGEND TO THE FIGURES

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**Table II:** Relative activities of the hybrid promoters (mSM22) evaluated in transient transfections *in vitro* into rabbit smooth muscle cells in primary culture (rabbit SMC), into ECV304 cells, into myoblasts C2C12, into HeLa cells, into NIH 3T3 cells, into carcinoma cells TU182, as well as into the renal cells 293. The relative activity of each promoter is expressed as a percentage of the luciferase activity obtained with the plasmid pCMV-leadTK. Enh-X: the enhancer sequence of hCMV-IE is cloned upstream of the X promoter in its normal orientation. HnE-X: the enhancer sequence of hCMV-IE is cloned upstream of the X promoter in the opposite orientation.

**Figure 1:** Schematic representations of the plasmids whose expression cassette contains the hSM $\alpha$ -actin hybrid promoter.

**Figure 2:** Schematic representations of the plasmids whose expression cassette contains the hybrid promoter mSM22 $\alpha$ .

**Figure 3:** Activities of the hybrid promoters evaluated in transient transfections *in vitro* into rabbit smooth muscle cells in primary culture (rabbit SMC), into endothelial cells derived from a human umbilical cord carcinoma (ECV304), into mouse myoblasts (C2C12) as well as into epithelial cells derived from a human cervical carcinoma (HeLa). The relative activity of each promoter is expressed as a percentage of the luciferase activity obtained with the plasmid pCMV-leadTK. Enh-X: the enhancer sequence of hCMV-IE is cloned upstream of the X promoter in its normal orientation. HnE-X: the enhancer sequence of hCMV-IE is cloned upstream of the X promoter in the opposite orientation.

**Figure 4:** Activities of the hybrid promoters evaluated in transient transfections *in vitro* into mouse embryonic fibroblasts (NIH 3T3), into cells derived from a human ENT carcinoma (TU182), as well as into transformed human embryonic renal cells (293). The relative activity of each promoter is expressed as a percentage of the luciferase activity obtained with the plasmid pCMV-leadTK. Enh-X: the enhancer sequence of hCMV-IE is cloned upstream of the X promoter in its normal orientation. HnE-X: the enhancer sequence of hCMV-IE is cloned upstream of the X promoter in the opposite orientation.

**Figure 5:** Activities of the hybrid promoters evaluated in gene transfer *in vivo* into the C57BL6 mouse cranial tibial muscle. The relative activity of each promoter is expressed as a percentage of the luciferase activity obtained with the plasmid pCMV-leadTK.

## MATERIALS AND METHODS

The methods conventionally used in molecular biology such as preparative extractions of plasmid DNA, centrifugation of plasmid DNA in cesium chloride gradient, agarose gel electrophoresis, purification of DNA fragments by electroelution, precipitation of plasmid DNA in saline medium with ethanol or isopropanol, transformation in *Escherichia coli* are well known to persons skilled in the art and are abundantly

described in the literature (Sambrook et al. "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989).

The plasmid pGL3-Basic, used for the cloning of the various promoter regions, is of commercial origin (Promega Corporation). The plasmids pCMV $\beta$  (Clontech Laboratories Inc.) and pUC18 (Boehringer Mannheim) are also of commercial origin.

The enzymatic amplification of DNA fragments by the PCR technique (Polymerase Chain Reaction) may be carried out using a DNA thermal cycler<sup>TM</sup> (Perkin Elmer Cetus) according to the manufacturer's recommendations.

The electroporation of plasmid DNA into *Escherichia coli* cells may be carried out with the aid of an electroporator (Bio-Rad) according to the manufacturer's recommendations.

The verification of the nucleotide sequences may be carried out by the method developed by Sanger et al. (*Proc. Natl. Acad. Sci. USA*, **74** (1977) 5463-5467) using the kit distributed by Applied Biosystems according to the manufacturer's recommendations.

## EXAMPLES

### EXAMPLE 1: Construction of hybrid promoters and of expression plasmids containing them

#### 1.1. Hybrid promoters hSM $\alpha$ -actin

• **Plasmid pHSMact.** The high-molecular-weight genomic DNA was prepared according to the method described by Sambrook et al. ("Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989) from a primary culture of human aortic smooth muscle cells (Clonetics).

This DNA was used as template for a first amplification by PCR using the following primers:

- Primer 6417 (5' GATGGTCCCTACTTATGCTGCTA 3') (SEQ ID 1) starting at position -1034 (promoter region) of the human specific smooth muscle  $\alpha$ -actin gene (Ueyama H. et al., *Mol. Cell. Biol.*, **4** (1984) 1073-1078. Access Genbank D00618).  
 - Primer 6418 (5' CTTCCATCATACCAAACTACATA 3') (SEQ ID 2) at position 1974 of the D00618 sequence situated inside the first intron of the hSMact gene. The reaction mixture comprises 1 mg of genomic DNA, 10 pmol of each of the two primers (6417 and 6418), 100 mM of each deoxyribonucleotide (dATP, dCTP, dGTP, dTTP), 2 mM MgCl<sub>2</sub> and 5 units of Taq DNA polymerase (PerkinElmer). The reaction volume is brought to 50 ml adjusted to the optimum PCR buffer concentration recommended by Perkin Elmer.

The PCR amplification is carried out in Micoamp<sup>TM</sup> tubes (Perkin Elmer) with the aid of a thermocycler PTC-100<sup>TM</sup> (MJ Research, Inc.). This amplification consists of a denaturing step at 95°C for 2 min followed by 30 cycles comprising a denaturing step of 15 sec at 95°C, an annealing step of 30 sec at 60°C and an extension step of 1 min at

72°C. These thirty cycles are followed by an additional extension of 5 min and then the PCR reactions are stored at 10°C.

- One microliter of this reaction was collected from this first reaction and then diluted in 10 ml of water. Next, 1 ml of this dilution was used to carry out a second
- 5 PCR under the same conditions as the first (above) but with a different pair of primers:
- Primer 6453 (5' CTGCTAAATTGctcgagGACAAATTAGACAAA 3') (SEQ ID 3), this primer introduces an XhoI site (underlined lower-case letters) upstream of the hSMact promoter (position -680).
  - Primer 6456 (5' CCCTGACAaagcctGGCTGGGCTGCTCCACTGG 3') (SEQ ID 4), this primer introduces an HindIII site at position +30 of hSMact.
- 10

After analysis on an agarose gel and then purification, the DNA fragment amplified by PCR is digested for 3 hours at 37°C with XhoI and HindIII and then cloned into the vector pGL3-Basic (Promega) previously digested with these same restriction enzymes, in order to generate the plasmid phSMact (Figure 1).

- 15 • **Plasmids pXL3130 and pXL3131.** A DNA fragment corresponding to the enhancer region of the promoter of the human cytomegalovirus IE (hCMV-IE) gene between positions -522 and -63 relative to the site of initiation of transcription was amplified by PCR using the plasmid pCMV $\beta$  as template and the oligonucleotides 8557 (5' ATC GAC GCG TGC CCG TTA CAT AAC TTA CGG 3') (SEQ ID 5) and 8558 (5' ATC
- 20 GAC GCG TCC GCT CGA GCG TCA ATG GGG CGG AGT TG 3') (SEQ ID 6) as primers. This fragment was digested with MluI and was then cloned into the plasmid phSMact previously digested with MluI and treated with alkaline phosphatase. Depending on the direction of insertion of the fragment, two different plasmids were obtained: pXL3130 and pXL3131. The schematic representations of these plasmids are assembled in the figure
- 25 (Figure 1). These plasmids contain, in the form of an MluI-NcoI fragment, a hybrid promoter consisting of the enhancer of the promoter of the hCMV-IE gene and of the promoter of the hSM $\alpha$ -actin gene.

## 1.2. Hybrid promoters mSM22

- 30 • **Plasmid pmSM22.** The high-molecular-weight genomic DNA was prepared according to the method described by Sambrook et al. ("Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989) from a Balbc mouse liver.

This DNA was used as template for a PCR amplification using the following primers:

- 35 - Primer 6517:  
(5' CCAGGCTGCActcgagACTAGTTCACCAACTCGA 3') (SEQ ID 7)  
this primer introduces an XhoI site (underlined lower-case letters) at position -436 of the promoter of the mouse SM22 alpha gene (Solway J. et al., *J. Biol. Chem.*, **270** (1995) 13460-13469; Access Genbank L41161).

- Primer 6518:

(5' TCGTTTGaagcttGGAAGGAGAGTAGCTTCGGTGTC 3') (SEQ ID 8), this primer introduces an HindIII site at position +43 of mSM22 alpha.

A reaction mixture comprising 1 mg of mouse genomic DNA, and 10 pmol of each of the two primers (6517 and 6518) was prepared with the same reagents as for hSMact and at the same concentrations, followed by a PCR amplification carried out under the same conditions (see Example 1.1.).

After analysis on an agarose gel and then purification, the DNA fragment amplified by PCR was digested for 3 hours at 37°C with XhoI and HindIII and then cloned into the vector pGL3-Basic (Promega) previously digested with these same restriction enzymes. The resulting plasmid was designated pmSM22 (Figure 2).

• **Plasmid pXL3152 and pXL3153.** A DNA fragment corresponding to the enhancer region of the promoter of the hCMV-IE gene between positions -522 and -63 relative to the site of initiation of transcription was amplified by PCR using the plasmid pCMV $\beta$  as template and the oligonucleotides 8557 (5' ATC GAC GCG TGC CCG TTA CAT AAC TTA CGG 3') (SEQ ID 5) and 8558 (5' ATC GAC GCG TCC GCT CGA GCG TCA ATG GGG CGG AGT TG 3') (SEQ ID 6) as primers. This fragment was digested with MluI and was then cloned into the plasmid pmSM22 previously digested with MluI and treated with alkaline phosphatase. Depending on the direction of insertion of the fragment, two different plasmids were obtained: pXL3152 and pXL3153. The schematic representations of these plasmids are assembled in Figure 2. These plasmids contain, in the form of an MluI-NcoI fragment, a hybrid promoter consisting of the enhancer of the promoter of the hCMV-IE gene and of the promoter of the mSM22 gene.

### 1.3. Control plasmid pCMV-leadTK

The expression vector pCGN previously described by Tanaka et al. (*Cell*, **60** (1990) 375-386) contains the CMV promoter (-522/+72) fused with the "leader" of the HSV tk gene (+51/+101) upstream of a sequence encoding the hemagglutinin epitope. The plasmid pCGN (10 ng) was used as template for a PCR amplification. The PCR reaction as well as the amplification were carried out under the same conditions as those used for hSMact and mSM22 (Examples 1.1 and 1.2). The primers which were used are the following:

- Primer 6718

(5' CCCGTTACATAACTTACGGTAAATGGCCCG 3') (SEQ ID 9), this primer hybridizes with the CMV promoter at position -522 (8 nucleotides downstream of the EcoRI site of pCGN).

- Primer 6719



(5' **g**GACGCGCTTCTACAAGGCGCTGGCCGAA 3') (SEQ ID 10), this primer hybridizes up to position 101 of the tk "leader". The first nucleotides G in bold is intended to restore the NcoI site of pGL3-Basic as will be clearly stated below.

The PCR fragment thus obtained is purified and then phosphorylated with the aid of T4 phage polynucleotide kinase (New England Biolabs). In parallel, the vector pGL3-Basic (Promega) was linearized with NcoI, purified and then treated with Klenow DNA polymerase (Boehringer Mannheim) in order to fill the NcoI site. This vector is then dephosphorylated with the aid of alkaline phosphatase (Boehringer Mannheim) and then used for the insertion of the phosphorylated PCR fragment. Thus, the guanosine (G) of the primer 6719 makes it possible to restore the NcoI site only when the CMV-tk leader fragment is oriented with the 5' part (primer 6718, position -522 of CMV) downstream of the HindIII site of pGL3-Basic and its 3' end (primer 6719, tk leader) is ligated into the NcoI site of pGL3-Basic (first ATG of luciferase). The plasmid thus obtained is designated pCMV-leadTK.

## EXAMPLE 2: Specificity of the hybrid promoters *in vitro*

This example illustrates the tissue specificity properties of the hybrid promoters of the invention *in vitro*.

### 2.1. Cell cultures

The rabbit smooth muscle cells (SMC) are cultured in DMEM<sup>TM</sup> medium (Life Technologies Inc.) supplemented with 20% fetal calf serum (FCS). The ECV304 cells are cultured in 199<sup>TM</sup> medium (Life Technologies Inc.) supplemented with 10% FCS. The C2C12 myoblasts, the HeLa cells, the NIH 3T3 cells as well as the TU182 cells are cultured in DMEM<sup>TM</sup> medium supplemented with 10% FCS. The 293 cells are cultured in MEM<sup>TM</sup> medium (Life Technologies Inc.) supplemented with pyruvate, nonessential amino acids and 10% FCS. All the cultures are carried out in an incubator at 37°C, in a humid atmosphere and under a CO<sub>2</sub> partial pressure of 5%.

### 2.2. Transfections *in vitro*

The transfections are carried out in 24-well plates and each transfection is carried out three times. Twenty-four hours before the transfection, the cells are inoculated: (i) at  $5 \times 10^4$  cells per well for the rabbit smooth muscle cells, the ECV304, NIH 3T3 and HeLa cells, (ii) at  $10^5$  cells per well for the TU182 cells, (iii) at  $3 \times 10^4$  cells per well for the C2C12 cells, and (iv) at  $2 \times 10^5$  cells per well for the 293 cells.

For each well, 500 ng of plasmid DNA (250 ng of plasmid of interest and 250 ng of pUC18) are mixed with the cationic lipid RPR120535 B (WO 97/18185) in an amount of 6 nmol of lipid per  $\mu$ g of DNA in DMEM<sup>TM</sup> medium (20  $\mu$ l final) comprising 150 mM of NaCl and 50 mM of bicarbonate. After 20 minutes at room temperature, the 20  $\mu$ l of the DNA/lipid mixture are brought into contact with the cells, in the absence of FCS, for 2 hours. The culture medium is then supplemented with FCS so as to obtain the percentage of FCS required for the culture of each cell type.

Forty-eight hours after the transfection, the culture medium is removed and the cells are rinsed twice with PBS (Life Technologies Inc.). The luciferase activity is then determined with the aid of the Luciferase Assay System™ kit (Promega Corporation) according to the manufacturer's recommendations.

### 5                   2.3. Specific activities of the hybrid promoters

The relative luciferase activities of the hybrid promoters (relative to the pCMV-lead TK promoter) measured *in vitro* in seven different cell types are assembled in Figures 3 and 4, as well as in Tables I and II. The results show that for the promoters hSMact (Table I) and mSM22 (Table II), the relative activity is a value which indeed  
10 reflects the specificity of these promoters for the smooth muscle cells; a specificity which has already been described in the literature (Skalli et al., *J. Histochem. Cytochem.*, **37** (1989) 315-321; Shimizu et al., *J. Biol. Chem.*, **270** (1995) 7631-7643; Li et al., *J. Cell Biol.*, **132** (1996) 849-859). Indeed, the relative activity in rabbit SMCs is at least 5 times higher than those observed in the other cell types: (i) from 5 to 20 times higher for the  
15 promoter hSMact (Table I), and (ii) from 5 to 25 times higher for the promoter mSM22 (Table II).

The results presented in Tables I and II show clearly that, in the smooth muscle cells, the activity of the four hybrid promoters according to the invention (Enh-hSMact, hnE-hSMact, Enh-mSM22 and hnE-mSM22) is comparable, in terms of  
20 strength, to that of the CMV promoter. Moreover, the relative activity of each of these promoters, in another cell type, is at least 10 times lower for the hSMact hybrid promoters, and at least 4 times lower for the mSM22 hybrid promoters, than that observed in the smooth muscle cells: (i) from 10 to 140 times for the hSMact hybrid promoters, and (ii) from 4 to 55 times for the mSM22 hybrid promoters. These hybrid promoters therefore  
25 preserve the same tissue specificity as that observed for the specific promoters hSMact and mSM22.

These results show, in addition, that the orientation of the enhancer region in the hybrid promoters of the invention has no significant influence on their activity.

The four hybrid promoters therefore possess, *in vitro*, activity in the  
30 smooth muscle cells which is as high as that for the CMV promoter (which is reputed to be a strong promoter), while preserving a tissue specificity which is comparable to, or even higher than, that of the promoters hSMact and mSM22.

### **EXAMPLE 3: Specificity of the hybrid promoters *in vivo***

This example illustrates the tissue specificity properties of the hybrid  
35 promoters of the invention *in vivo*.

#### **3.1. Gene transfer into the skeletal muscle**

The various plasmids were injected, intramuscularly, into the cranial tibial muscle of 5-week old female C57BL6 mice. Each plasmid, diluted in a solution of NaCl at 150 mM final, is injected in an amount of 10 µg per muscle. Three days after injection, the

muscles are collected in 2 ml of Cell Culture Lysis Reagent™ buffer (Promega Corporation), and ground with the aid of a Diox homogenizer (Heidolph). The ground product is then centrifuged for 15 minutes at 4000 g, and then the luciferase activity is evaluated with the aid of the Luciferase Assay System™ kit (Promega Corporation) according to the manufacturer's recommendations.

### 3.2. Activities of the hybrid promoters in the skeletal muscle *in vivo*

The relative activities of the two specific promoters (hSMact and mSM22) as well as those of two of the hybrid promoters of the invention (Enh-hSMact and Enh-mSM22) were also evaluated *in vivo* after transfer of naked DNA into the mouse cranial tibial muscle. The results assembled in Figure 5 show that the activity of the Enh-hSMact promoter is 100 times lower than that of the CMV promoter. Likewise, the activity of the Enh-mSM22 promoter is 17 times lower than that of the CMV promoter. Thus, the tissue specificity observed *in vitro*, and in particular in the C2C12 cells which constitute the model which is closest to that used *in vivo*, is therefore conserved *in vivo*.

### EXAMPLE 4: Construction of recombinant adenoviruses expressing the GAX protein under the control of specific hybrid promoters

This example is intended to describe an adenoviral vector carrying the gene encoding the GAX protein operably linked to the hybrid promoter of the invention composed of the CMV enhancer and the SM $\alpha$ -actin promoter (enh-hSMact).

The human *gax* gene contains 912 base pairs and encodes a transcription factor of 303 amino acids which is involved in the arrest of cell growth (growth-arrest-specific homeobox) and which has a role on the proliferation of the human smooth muscle cells. This homeodomain-containing gene was initially isolated from the aorta and is expressed in particular in adult cardiovascular tissues (Gorski et al. 1993).

The sequence of the human *gax* gene has been to clone from a skeletal muscle cDNA library by PCR (Polymerase Chain Reaction) using, as primer, a sequence derived from the human *gax* gene and published by Walsh et al. (Genomics (1994), 24, p535). The sequence was then cloned into the expression vector pXL3297. This plasmid is derived from the plasmid Bluescript (Stratagene) containing the human CMV IE enhancer/promoter (-522/+72) (Cell (1985), 41, p521) and the SV 40 poly A (2538-2759) (GenBank SV4CG locus).

The construction uses the plasmid pXL3130 described in Example 1 (Figure 1) into which the SM $\alpha$ -actin promoter had been previously introduced. The plasmid pXL3297 is an expression vector containing the human *gax* gene. It was digested with the enzymes HindIII and AvrII in order to introduce the human *gax* gene into the previous plasmid pXL3282 also digested with the enzymes HindIII and AvrII in order to give the plasmid pXL 3300. As indicated in Figure 6, in which the various steps of the construction of the plasmids described above are detailed, the final plasmid, pXL3310, contains an expression cassette consisting of the CMV-IE enhancer, the SM $\alpha$ -actin promoter (pSMA),

combined according to the invention and operably linked to the gene encoding the human GAX protein as well as the SV40 poly A termination signal.

The human gax gene expression cassette is then introduced into a recombinant human adenovirus of serotype 5 (Ad5) deleted for the E1 and E3 regions by co-transfection and homologous recombination between the plasmid carrying the cassette for expressing the gax gene and the adenovirus, in encapsidation cells. These cells are preferably the 293 line. The production of an adenovirus stock containing the cassette for expressing the human gax gene results from the lysis of the encapsidation cells 2 or 3 days after the infection and the isolation of the recombinant viral particles by cesium chloride gradient centrifugation.

The viral particles are then used to study the expression of the human gax gene under the control of the promoter of the invention in smooth muscle cells.

The expression of the GAX protein is checked 24 hours after the infection of the primary smooth muscle cells by immunofluorescence or by western blotting using the rabbit anti-gax polyclonal antibodies.

The expression of the messenger RNAs is analyzed 24 hours after the infection of the smooth muscle cells by dot blot and northern blotting using an oligonucleotide whose sequence is present in the gax gene.

The analysis of the biological activity of the adenovirus encoding the gax gene is carried out in the following manner:

Smooth muscle cells at the exponential growth phase are infected with an adenovirus containing the gene encoding the GAX protein under the control of the enh-hSMact promoter in the absence and in the presence of 125 ng of lipofectin (48-well plates). The adenoviruses (in variable dilutions) and the lipofectamine are incubated for 30 minutes at room temperature in a serum-free medium. The mixture or the virus alone is brought into contact with the cells for one hour at 37°C. At the end of the period of infection, the medium containing the virus is removed and the cells are incubated in DMEM medium containing 0.5% SVF. Within the 24 hours following the infection, the culture medium is replaced with a growth medium for half of the cultures and the incubation is continued for 48 hours to allow the cells to enter the S phase. For the other half of the cultures, a weakly mitogenic medium is added in order to maintain the cells in quiescence. The viable cells are counted 72 hours after the infection using the Alamar protocol.

**TABLE I**

	Plasmids (Promoters)				
	without plasmid (-)	pCMV-leadTK (CMV-leadTK)	phSMact (hSMact)	pXL3130 (Enh-hSMact)	pXL3131 (hnE-hSMact)
Rabbit SMC	< 0.01	100.00	0.67 ± 0.06	107.10 ± 11.23	77.9 ± 7.93
ECV304	< 0.01	100.00	0.12 ± 0.05	5.19 ± 0.99	4.68 ± 0.77
C2C12	< 0.01	100.00	0.11 ± 0.01	4.48 ± 0.28	2.81 ± 0.09
HeLa	< 0.01	100.00	0.03 ± 0.01	10.31 ± 1.13	8.00 ± 0.19
NIH 3T3	< 0.01	100.00	0.08 ± 0.01	4.25 ± 0.08	3.66 ± 0.28
TU182	< 0.01	100.00	0.03 ± 0.00	6.15 ± 0.47	8.46 ± 0.41
293	< 0.01	100.00	0.04 ± 0.00	0.77 ± 0.07	0.72 ± 0.07

**TABLE II**

	Plasmids (Promoters)				
	without plasmid (-)	pCMV-leadTK (CMV-leadTK)	pmSM22 (mSM22)	pXL3152 (Enh-mSM22)	pXL3153 (hnE-hSM22)
Rabbit SMC	< 0.01	100.00	2.13 ± 0.29	94.61 ± 13.43	100.21 ± 23.20
ECV304	< 0.01	100.00	0.42 ± 0.13	13.81 ± 0.35	11.83 ± 1.95
C2C12	< 0.01	100.00	0.20 ± 0.01	11.19 ± 1.84	8.37 ± 0.08
HeLa	< 0.01	100.00	0.04 ± 0.01	12.87 ± 1.04	15.90 ± 1.92
NIH 3T3	< 0.01	100.00	0.32 ± 0.01	9.49 ± 0.41	9.59 ± 0.53
TU182	< 0.01	100.00	0.08 ± 0.01	25.78 ± 3.04	26.37 ± 0.98
293	< 0.01	100.00	0.09 ± 0.00	2.29 ± 0.02	1.78 ± 0.03

5

**CLAIMS**

1. Hybrid promoter comprising:
  - all or part of the enhancer region of a strong and ubiquitous promoter/enhancer, and
  - 10 - a promoter region allowing specific expression in the smooth muscle cells.
2. Hybrid promoter according to claim 1, characterized in that the enhancer region is chosen from the enhancer region of the cytomegalovirus immediate-early (CMV-IE) gene, the enhancer region of the rous sarcoma virus LTR (RSV-LTR), the enhancer region of the SV40 virus, and the enhancer region of the EF1 $\alpha$  gene.
- 15 3. Hybrid promoter according to claim 2, characterized in that the enhancer region is the enhancer region of the immediate-early gene of the cytomegalovirus (CMV-IE), preferably of the human cytomegalovirus (hCMV-IE).
4. Hybrid promoter according to claim 1, characterized in that the promoter region comprises all or part of the promoter of the gene encoding the  $\alpha$ -actin of smooth muscle cells (SMact) or of the SM22 gene.
- 20 5. Hybrid promoter comprising:
  - all or part of the enhancer region of the human cytomegalovirus immediate-early (hCMV-IE) gene, and
  - 25 - all or part of the promoter of the gene encoding the  $\alpha$ -actin of smooth muscle cells (SMact).
6. Hybrid promoter comprising:
  - all or part of the enhancer region of the human cytomegalovirus immediate-early (hCMV-IE) gene, and
  - 30 - all or part of the promoter of the SM22 gene.
7. Hybrid promoter according to claim 1, characterized in that the promoter region comprises a basal promoter and a sequence conferring tissue specificity, said sequence being derived from the SMact promoter and/or from the SM22 promoter.
8. Expression cassette comprising a nucleic acid encoding an RNA or a polypeptide of interest, placed under the control of a hybrid promoter according to one of claims 1 to 7.
- 35 9. Cassette according to claim 8, characterized in that it comprises, in addition, a signal for termination of transcription.
10. Cassette according to claim 8 or 9, characterized in that the nucleic acid encodes a protein chosen from the proteins involved in the cell cycle, the proteins inducing apoptosis, the proteins capable of modifying the proliferation of the smooth muscle cells, the proteins inducing angiogenesis and the transcription factors.
- 40

- 5                    11.      Vector comprising a hybrid promoter according to claim 1 or a cassette according to claim 8.
12.      Vector according to claim 11, characterized in that it is a plasmid, a cosmid or any DNA not encapsidated by a virus.
13.      Vector according to claim 11, characterized in that it is a
- 10 recombinant virus, preferably derived from an adenovirus, a retrovirus, a herpesvirus or an adeno-associated virus.
14.      Composition comprising a vector according to claim 12 and a chemical or biochemical transfer agent.
15.      Composition comprising a recombinant virus according to
- 15 claim 13 and a physiologically acceptable vehicle.
16.      Cell modified by a cassette according to claim 8 or a vector according to claim 11.
17.      Use of a hybrid promoter according to one of claims 1 to 7 for the preparation of a composition intended for the selective expression of a nucleic acid in the
- 20 smooth muscle cells.
18.      Use of a hybrid promoter according to one of claims 1 to 7 for the preparation of a composition intended for the expression of a nucleic acid in the smooth muscle cells and not in the endothelial cells which are found in the vicinity of the blood vessel.



ST98032

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5

Abstract

- 10 The invention concerns a hybrid promoter comprising all or part of the enhancer region of a strong and ubiquitous promoter/enhancer; a promoter region enabling specific expression in smooth muscle cells, and all vector or cell containing said promoter, and their uses.

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(54) Titre: UTILISATION DE PROMOTEURS SPECIFIQUES HYBRIDES POUR CONTROLER L'EXPRESSION TISSULAIRE

(57) Abstract

The invention concerns a hybrid promoter comprising: all or part of the enhancer region of a strong and ubiquitous promoter/enhancer; a promoter region enabling specific expression in smooth muscle cells, and all vector or cell containing said promoter, and their uses.

(57) Abrégé

La présente invention concerne un promoteur hybride comprenant: tout ou partie de la région enhancer d'un promoteur/enhancer fort et ubiquitaire, et une région promoteur permettant l'expression spécifique dans les cellules musculaires lisses, ainsi que tout vecteur et cellule contenant ce promoteur, et leurs utilisations.

UNITED STATES OF AMERICA

# DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor(s), I/We hereby declare that:

My/Our residence(s), post office address(es) and citizenship(s) are as stated below my name(s).  
I/We verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

## USE OF SPECIFIC HYBRID PROMOTERS FOR CONTROLLING TISSUE EXPRESSION

and the specification of which ☐ is attached hereto (Attorney Docket No. ST98032)  
(check one) ☒ was filed on **March 23, 2001** as U.S. Application Number **09/787,995**  
and was amended on (if applicable).  
☐ was described and claimed in PCT Int'l Application Number filed on  
and as amended under PCT Article 19 on (if any).

I/We hereby state that I/We have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to above.

I/We acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 C.F.R. 1.56.

☒ I/We hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or 365 (b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one other country other than the United States of America, listed below and having a filing date before that of the application on which priority is claimed. I/We have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Foreign	<b>FR 98 12000</b>	<b>France</b>	<b>25 September 1998</b>
Priority:	Number	Country	Day/Month/Year Filed

Prior	<b>Number</b>	<b>Country</b>	<b>Day/Month/Year Filed</b>
Foreign			
Appln(s):			

☒ I/We hereby claim the benefit under Title 35, United States Code §119(e) of any United States Provisional application(s) listed below:

<b>US60/123,298</b>	<b>04 March 1999</b>
Number	Filing Date

☒ I/We hereby claim the benefit under Title 35, United States Code §120 or 365(c) of any United States application(s) or international application designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I/We acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

<b>PCT/FR99/02265</b>	<b>23 September 1999</b>	<b>Completed</b>
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